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COMPARATIVE STUDIES OF DIPLOCOCCI DECOLORIZED BY GRAM'S METHOD, OBTAINED FROM THE SPINAL FLUID AND FROM THE NARES OF CASES OF EPIDEMIC CEREBROSPINAL MENINGITIS.

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THE studies which are here epitomized were undertaken as a part of the work of the Commission for the Study of Cerebrospinal Meningitis appointed by the Department of Health of the City of New York in March, 1905. The Commission deemed it important to ascertain whether microorganisms identical with those occurring in the cerebrospinal cavity were to be found in the upper air passages of those suffering from epidemic meningitis; and a part of the cultural work for the Commission was the isolation, from the nares of such cases, of diplococci not readily distinguishable from the diplococcus of meningitis by morphological characters.

Pure cultures of the Diplococcus intracellularis (Weichselbaum) were secured from the spinal fluid obtained by lumbar puncture from 50 cases of epidemic cerebrospinal meningitis and served for comparison with the cultures from the nares.

CULTURES.

The organisms from the nose or throat were obtained from streak-plate cultures made from the secretions collected with sterile cotton swabs. The medium employed for these plate cultures was 2 per cent glucose agar, enriched with ascitic fluid or sheep serum mixed with the melted agar at the time the plates were poured, or by distributing a little human or rabbit blood upon the surface of the agar after it had solidified. Cultures from the spinal fluid were obtained upon similar agar plates. Subcultures were made upon slants of 2 per cent glucose agar with or without other addition, or with a little blood or sheep serum upon the surface. The cultures on 2 per cent glucose agar without enrichment have usually exhibited excellent growth, provided the medium was protected from drying. Even the slight drying due to loose cotton plugs has greatly impaired the growth; but when the plugs were tight, so that the surface of the medium was kept in a moist atmosphere, abundant growth could almost always be secured, and the cultures remained alive for a comparatively long time. To this end the air in the thermostat was also kept moist by placing a dish of water on the lowest shelf.

The cultures from spinal fluids upon 2 per cent glucose agar are gray and moist, usually only slightly raised above the surface of the medium, and appear to be strictly confined to those portions of the medium that were directly inoculated, there being no tendency to spread upon its surface. The condensation water becomes cloudy, with the formation of an abundant sediment, and, frequently, a slight pellicle is perceptible upon the surface of the condensation water. In cultures that are three or four days old the growth has a slight mucinous consistency, but is easily removed from the surface of the agar with a platinum loop. When scraped from the agar, the mass of bacteria has a pinkish buff color, and is readily broken up and disseminated in water or saline solution. A considerable variation in the luxuriance of growth was noted in many of the subcultures from different spinal fluids. In some cases a much more abundant inoculation of fresh media was necessary for success than in others, and the resulting growth was in the form of discrete colonies rather than a uniform layer. In other cases the growth was so abundant and formed such a thick layer upon the media as to excite the suspicion that the cultures were contaminated. These variations in growth depend not merely upon the particular strains under observation, but also upon the media employed. Slight indeterminate variations in the latter affected the luxuriance of growth very markedly. In two or three instances a particular lot of glucose agar was found to be unfit for use, even an abundant inoculation yielding no growth, although the preparation of the media could not have differed greatly from that employed when the result was most favorable.

An explanation of these experiences cannot be given; the method of preparing the medium had become an established routine from which there were not conscious departures.

Owing to the variations noted in the cultures on 2 per cent glucose agar, it is impossible to formulate a typical description of the growth of the D. intracellularis on this medium that would serve for its certain identification.

Notwithstanding this fact, the cultures of diplococci decolorized by Gram's method of staining and obtained from the upper air passages could, in many instances, be readily distinguished from D. intracellularis when grown on 2 per cent glucose agar. These easily recognized as differing from this diplococcus were divisible into three groups:

1. Those yielding very luxuriant, heavy, mucinous growths of a gray color and forming a much thicker layer upon the agar than those of D. intracellularis. These cultures readily disseminated in saline solution and yielded a good suspension upon filtration through paper. They failed, however, to agglutinate in very low dilutions (1:20 or 1:50) of serum from a horse immunized with D. intracellularis from spinal fluids, while the latter was agglutinated in dilutions of 1:500 to 1:1000.

2. Those giving rise to very coherent growths that adhered strongly to the surface of the agar, so that it was extremely difficult to remove any considerable quantity of the growth with a platinum loop. This growth did not disseminate easily in saline solutions and was almost entirely removed by filtration through filter paper, so that satisfactory suspensions could not be obtained for agglutination tests.

3. Cultures which, within one to three days, produced a greenish-yellow fluorescence in the agar.

After eliminating these three groups, there remained:

4. A group of cultures which could not be readily distinguished by the gross appearances of the growth on 2 per cent glucose agar from cultures of D. intracellularis on the same medium. This group was of greater interest in the present inquiry than the other three and received most study. It could be divided into two subgroups: those more closely resembling Micrococcus catarrhalis, and those more closely resembling, if not identical with, D. intracellularis. These two subgroups may be provisionally designated as catarrhalis-like and intracellularis-like groups. The former were certainly not all of the same species, and it is doubtful whether any of them were identical with M. catarrhalis of Ghon and Pfeiffer. The intracellularis-

like group of diplococci appeared to be quite homogeneous, and there is reason to believe that they were identical with D. intracellularis of Weichselbaum. Coverglass preparations of the catarrhalis-like diplococci show somewhat less variations in size, form, and intensity of stain than similar preparations of D. intracellularis from spinal fluids, but these differences do not appear marked enough to inspire confidence in them as a means of distinguishing with certainty these diplococci from D. intracellularis. There was, however, a difference in the suspensions in 0.75 per cent solutions of salt. The organisms from the cultures of catarrhalis-like cocci showed a distinct tendency to settle and form a sediment within a few hours (although they readily passed through filter paper), while those of the intracellularis-like cocci remained in suspension for a very much longer time. This difference was constant. Within 24 hours a suspension of the growth from an agar culture of the former, one day old and containing enough organisms to render it distinctly opalescent, would yield a marked sediment when kept at rest over night in one of the tubes used for macroscopic agglutinations. Suspensions of D. intracellularis of like density failed to exhibit such sedimentation. In one instance such a suspension was kept in the ice-box for three weeks without more than a trifling sedimentation. These differences rendered macroscopic agglutinations of the catarrhalis-like group, for purposes of comparison, extremely difficult. Further differences, revealed by the readiness with which sera containing catarrhalis-like or intracellularis cocci could be filtered through Berkefeld filters, will be referred to later.

AGGLUTINATIONS.

Comparison of the various cultures of the fourth group and D. intracellularis was attempted with the use of macroscopic agglutination tests. For each test 1 c.c. of the serum properly diluted was mixed with 1 c.c. of a suspension of the organism in 0.75 per cent salt solution. In nearly all of these studies the final dilutions of the sera in each series of tests were: 1:20, 1:50, 1:100, 1:200, 1:1000, and 1:2,000. In a few cases higher dilutions were made, but did not add to the value of the results. The mixtures of serum, saline solution, and bacterial suspensions were placed in the incubator (37°) for three hours and then in the ice-box (about 5°) over night. At the end of three or even six hours there was usually no well-marked evidence of agglutination, but after 24 hours the lowest dilutions showed distinct clumping. The first routine observations were made after from 15 to 24 hours, and a second observation from 42 to 48 hours after the tests were placed in the incubator. In the majority of instances the second observation revealed agglutination in one or two of the dilutions next higher than those giving evidence of this reaction at the first observation. The time which was allowed to elapse before making the observations is much longer than the usual limit adhered to in making macroscopical tests on agglutination. But it is believed that no errors due to growth of the organisms could vitiate the results. It is certain, from experiments made to determine this fact, that much more rapid reactions might have been secured if broth cultures of D. intracellularis had been used instead of suspensions from agar cultures. The saline suspensions were preferred, because it was believed that more uniform results could in this way be obtained in making tests at different times with an organism so capricious in vigor of growth as is intracellularis Suspensions of apparently identical richness could be prepared without great difficulty at any time. The delay in reaction appeared to be relatively unimportant. In some of the tests ¼ per cent of carbolic acid was added to the salt solution to preclude growth of the organisms. This addition did not in any way modify the results.

The sera employed in these agglutination tests were either from patients suffering from epidemic cerebrospinal meningitis or convalescent from this disease, or from animals which had been immunized with either single or repeated injections of large quantities of D. intracellularis obtained from spinal fluids. The animals used were rabbits, a goat, a horse, and several geese. The animals usually bore the injections of cultures from spinal fluids well. The most susceptible were the rabbits. But these soon acquired a considerable tolerance for even very large quantities, manifesting very slight, if any, acute symptoms, though they gradually became greatly emaciated, and lost much hair when the injections were continued for a long time. The geese were very tolerant of D. intracellularis. In one case about 5 c.c. of a thick purée prepared from 14 plate cultures (each about 10 inches in diameter) incubated for 24 hours, was injected into the pectoral muscles of a goose without marked effect either locally or in the general condition. The animal refused food for a few hours, but on the next day ate as usual. The cultures of catarrhalis-like cocci and other diplococci, certainly not meningococci, from the nose and throat were often much more virulent for geese and rabbits when given subcutaneously or intraperitoneally, the first injection frequently causing death.

The goat was immunized by Dr. Simon Flexner, who kindly furnished the serum for these tests. Large quantities of D. intracellularis from the spinal fluids obtained from 13 cases of epidemic meningitis were used by him to immunize this animal, and the injections were repeatedly given during a period of many weeks.

The horse serum came from an animal immunized with large quantities of dead cultures at the Research Laboratory of the New York Department of Health, and was generously contributed by Dr. Park. Some of the sera were preserved with chloroform, trikresol, toluol, or $\frac{1}{4}$ per cent phenol; others received no preservative, but were collected and kept uncontaminated. Comparative tests failed to reveal any influence exerted by these additions upon the agglutinating power of the sera.

None of these sera showed a high specificity in their agglutinations of D. intracellularis. They agglutinated in dilutions from 1:200 to 1:4,000, or two to ten times as great as the sera from normal animals of the same species. But it is difficult to utilize even this moderate increase in agglutinating power, because the conditions under which the tests were made differed unavoidably at different times, and even slight variations in condition influence the limits of the dilution at which positive results are obtained in a very great degree. The density of the emulsion, for example, was found to make a very great difference in the development of distinct agglutination in the higher dilutions. This fact is probably related to the observation that a complete agglutination of all the organisms in an emulsion, with a sedimentation of the resulting clumps, rarely takes place when the macroscopic method and saline suspensions are used. Free organisms are almost always present in sufficient abundance to render the fluid perceptibly opalescent, even when the agglutination of the remainder of the organisms gives rise to relatively large and heavy clumps that settle to the bottom of the liquid. Complete agglutinations are, therefore, comparatively rare when this method is employed. They appear to be much more common, especially in the lower dilutions, when broth cultures are used in place of suspensions in normal saline solution.

The fact, already mentioned, that the suspensions of catarrhalis-like diplococci tended to form a distinct sediment within 24 hours, rendered a comparison of the agglutinating power of the sera upon this group of organisms with the agglutination of intracellularis cocci practically impossible, but this sedimentation itself furnished a means of distinguishing these cultures from those of the latter organism. The difference between an agglutination with settling of the clumps and a sedimentation of the individual organisms is readily demonstrable upon moderate amplification. For this purpose a microscope with the tube in a horizontal position and a Leitz No. 3 objective were used to control the macroscopic observations. By rotating and then suddenly tilting the agglutination tube the sediment could be thrown up from the bottom along the side of the tube and readily examined with the microscope. The latter was placed in front of a window and the mirror removed. A very small diaphragm was employed, and the individual bacteria could be distinguished without difficulty. This microscopical control, therefore, served also to eliminate contamination with organisms morphologically differing in marked degree from D. intracellularis which might occasion errors in the observations.

The foregoing observations suffice to show the futility of an attempt to use these methods of agglutination in the comparative study of *all* the diplococci decolorized by Gram's stain found in the nose and throat, and those obtained from spinal fluids. In some cases they simply demonstrate constant differences in the suspensions of different cultures.

It was thought that more obviously biological differences or likenesses of general applicability might be revealed if the various agglutinins in the sera were removed by absorption with large quantities of a given species, and the serum thus deprived of one set of agglu tinins applied to suspensions of the various organisms under study, with a view to determining which of these species were then agglutinated. Many such experiments were made, with apparently gratifying success, but a critical review of the results casts much doubt upon their significance and also reveals unexpected difficulties in technique, which have not yet been overcome.

It was found that D. intracellularis from spinal fluids apparently removed all the agglutinins capable of clumping the organisms derived from the spinal fluids and also, as far as could be determined, those agglutinating the catarrhalis-like organism and other members of the fourth group from the nares; whereas the latter failed to remove from the serum of animals immunized with D. intracellularis more than a fraction of the agglutinins clumping D. intracellularis. But the very heavy suspensions necessarily used to insure absorption of the agglutinins were very different in the two cases. It was comparatively easy to separate the catarrhalis-like organisms from the diluted serum (usually diluted 1 in 5 or 1 in 10 with normal salt solution) either by centrifugalizing or by filtration through a Berkefeld filter. But it was found to be impossible to free the serum sufficiently from D. intracellularis with any available centrifuge. Although most of the organisms were thrown down, so large a number remained in suspension that the use of the serum was very unsatisfactory, especially in view of the delayed reactions already described. The separation of the serum by filtration through a Berkefeld filter, while successful, was exceedingly slow in comparison with the similar filtration when cultures of the catarrhalis-like cocci were employed. The heavy suspensions of D. intracellularis quickly formed a dense coating upon the surface of the filter, while an equally dense suspension of the catarrhalis-like organisms was more granular, or less slimy, and the deposit upon the filter impeded the passage of the serum in a very much less degree. This difference in the physical character of the two suspensions, which persisted in considerable, though less degree, even when the centrifuge was used before filtration, excited the suspicion that the agglutinins of the serum were mechanically held back by the coating of organisms or slimy substances in the suspension, and this suspicion was increased by the observation that a heavy suspension in diluted immune serum of a culture from the throat, which was certainly neither D. intracellularis nor one of the catarrhalis-like group, but which produced much mucinous material on 2 per cent glucose agar, also filtered exceedingly slowly and removed nearly if not quite all the agglutinins clumping D. intracellularis and intracellularis-like diplococci. If the loss of agglutinins were due to a mechanical prevention of their passage through the clogged filter and not to a union with the organisms, the value of these absorption tests, when all the agglutinins have been removed, is so seriously impaired as to cast doubt on their having any specific value, though they do reveal differences in the cultures used for absorption and, for this reason, are not without significance.

To test the effect of clogging a Berkefeld filter with a colloid so as to lengthen the time required for the passage of a diluted serum, a 1 per cent colloidal solution of corn starch boiled in 0.75 per cent salt solution was used as a diluent of the immune serum, and compared with a similar dilution with normal salt solution used as a control. The latter passed through a Berkefeld filter in 15 minutes. The starch mixture required about 20 hours, or 80 times as long. The agglutinating limits of the starch filtrate upon various of the organisms under study was found to be only $\frac{1}{4}$ to $\frac{1}{10}$ that of the control. This result appears to support the idea that the agglutinins might be mechanically held back by the coating upon the filter when intracellularis was used for absorption. It is possible, however, that the colloidal starch may have had some direct action upon the agglutinins in the serum. But this might also be the case with collodial (slimy) substances in the cultures of D. intracellularis. That the agglutinins may in this case be mechanically separated by filtration is also suggested by an observation indicating that the removal of the major part of the diplococci from the diluted serum with the centrifuge somewhat hastens subsequent filtration, but furnishes a filtrate with greater agglutinating power than it possessed when filtration was slower.

Notwithstanding the fact that these absorption tests have failed to establish a definite union between specific agglutinins in the immune sera and the intracellularis organisms, they throw some light on the relations between D. intracellularis and the diplococci of the fourth group found in the nose and throat. Some of the latter were identical in behavior with D. intracellularis; others yielded suspensions much more readily filtered or freed from the organisms with the centrifuge.

FERMENTATION.

A more striking biological distinction between D. intracellularis and the catarrhalis-like group of cultures was the differences they exhibited in the production of acid when grown in a suitable medium containing dextrose. Twenty-six of the cultures from the spinal fluid, three from the blood, and sixteen from the nose or throat of patients with epidemic meningitis were studied with a view to determining the production of acid when grown in the presence of dextrose.

The medium employed was a mixture of nutrient broth containing 1 per cent dextrose, sheep serum diluted with three times its volume of water, and 1 per cent solution of Kahlbaum's or Merck's purified litmus. These three constituents were separately sterilized in the Arnold sterilizer on three successive days, and then one part of the diluted serum mixed with three parts of the broth and enough of the litmus solution added to impart a distinct color to the whole. The mixture thus prepared was incubated for two to three days to detect accidental contamination. All of the cultures of D. intracellularis from spinal fluids and all of the cultures from the blood produced an unmistakable acid reaction in this medium after incubation at 37° for 24 hours. The intensity of this reaction usually increased slightly during the next 24 to 48 hours, but in no case was the acid production sufficiently abundant to occasion coagulation in the medium. When the reaction has reached a certain degree of acidity it appears to remain about constant, and to render the medium unfavorable for the further growth of the organisms. At the end of nine days only three out of 29 such cultures were found to contain living organisms. Similar cultures made with the organisms from the nose and throat differentiated them into three groups: (1) those which produced acidity without coagulation, i. e., the same change wrought by D. intracellularis; (2) those causing the formation of acid with coagulation in 24 hours; and (3) those producing no acidity (or, perhaps causing an alkaline change of reaction) even after nine days' incubation.

Of the 16 cultures from the nose and throat, six affected this broth-serum-glucose medium in exactly the same way as D. intracellularis. Of these six cultures a few had already been used for observations concerning their susceptibility to agglutination and their ability to absorb agglutinins from immune sera. In all these respects and in the characters of the suspensions they furnished, and in their various cultures, they corresponded to parallel observations on D. intracellularis. As far as our knowledge can justify a conclusion, these cultures were identical with this diplococcus and the latter were, therefore, present in the upper air passages in the cases of epidemic meningitis from which these cultures were obtained.

Most of the work furnishing the results here summarized was done during the summer of 1905 with the assistance of Mr. Hubert C. Ward, to whom grateful acknowledgment for many helpful suggestions and unflagging interest is cordially extended.